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TITLE: ISOLATION, SCREENING AND CHERACTERIZATION OF CADMIUM RESISTANT BACTERIA FOR BIOREMEDIATION

Short synopsis

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ABSTRACT

Environmental pollution due to anthropogenic activities and natural sources is increasing at a rapid rate. Heavy metals such as cadmium, nickel, lead, chromium, arsenic etc. are well-known components of industrial effluents which are discharged into the environment and consequently pollute the ecosystem. The presence of these heavy metals in the environment has been a subject of great concern due to their toxicity, non-biodegradable nature and long biological half-lives preventing their elimination from biological tissues. Several technologies exist for the removal of metal contamination like precipitation, cementation, ion-exchange and reverse osmosis. This process simply transfers the pollutants, creating a new waste such as incineration residues and does not eliminate the problem. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activities. As such, it is easy to operate, does not produce secondary pollution and show higher efficiency at low metal concentration. Bioremediation uses biological agents, mainly microorganisms (yeast, fungi or bacteria) to clean up contaminated soil and water. The isolation of heavy metal resistant bacteria and the understanding of the mechanisms they use in order to remove the heavy metals may contribute to the development of improved detoxification processes. This investigation is focused on the isolation, screening and characterization of cadmium resistant bacteria from soil sediment for environmental cleanup.

Keywords: soil sample, bacterial isolates, heavy metals, microbial remediation.

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INTRODUCTION

Rapid industrialization has substantially increased the disposal of heavy metals and radio-nuclides from certain industries into the environment. There are released into the environment through effluents from industries such as electroplating, paint pigment, electrical accumulators and batteries (Kok *et al*., 2001). Additionally, the agricultural activities like application of agrochemicals and sewage in agricultural fields also add a substantial amount of metals in the soils (Rajaganapathy *et al.,* 2011). The metal pollution is of great concern as these hazardous pollutants are accumulated in living organisms including plants, animals, microorganisms and human (Hashem and Abed, 2002) and are accountable for many metabolic and physiological disorders (Matyar *et al.*,2010).

The term heavy metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentration. Metals play a vital role in the metabolic processes of the biota. Some of the heavy metals are necessary and are essential to the organisms as micro nutrients (cobalt, manganese, chromium, nickel, iron, and zinc *etc*.) and are known as trace elements. They act as catalysts in enzymatic reactions and also help in regulating osmotic balance. On the other hand, some other heavy metals have no biological role and are toxic to the organisms even at very low concentration (cadmium, mercury, lead *etc*.). Cadmium is one of the heavy metals, which is highly toxic to human, plants and animals. It occurs naturally in the environment by the erosion and abrasion of rocks and soils, and from events such as forest fires and volcanic eruptions. It gets released into the environment by various human activities such as mining, smelting, incineration of plastics and batteries, burning of fossil fuels etc (Tang *et al.,* 2006). All over the world cadmium contaminated waste water and effluents are being generated either directly due to cadmium production or through secondary sources. Cadmium has a half-life of 10-30 years in the human body. Therefore, its toxic effects are especially pronounced in animals of higher trophic levels, particularly in humans. It causes bone diseases, kidney damage and cancer. Chronic exposure of cadmium is known to cause renal dysfunction, bone degeneration, liver damage, and blood damage (Nies, 2003). Cadmium may also battle with other metals such as zinc and selenium for enclosure into metallo-enzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin. In prolonged cadmium exposure, disorders of calcium metabolism occur, causing osteomalacia (Fauci *et al.,* 1998; Williams *et al*., 1999). This leads to painful fractures,

hence the name given to the chronic exposure disease in Japan: *Itai-itai* disease (literally "ouch!-ouch!" disease; Williams *et al.*, 1999; 1998; Taylor & Francis, 2003). The metal is of particular concern because it is non-degradable and consequently persistent.

Removal of cadmium from effluents before they are discharged into the environment can be accomplished by processes such as, chemical precipitation, cementation, solvent extraction, reverse osmosis and ion exchange (Meena & Rajagopal, 2003). Application of such methods however is sometimes restricted because of technical or economical constraints (Al-Garni, 2005). Bioremediation by microorganisms promises to be an excellent alternative. Awareness in the application of bioremediation for environmental protection efforts has improved within the last decade because it is easy to operate, does not create secondary pollution and show higher efficiency at low metal concentrations. Many bacteria have been isolated that are highly resistant to many heavy metals. Resistance to heavy metals can be due to one or more of several mechanisms. These mechanisms include the formation and sequestration of heavy metal, reduction of metal to a less toxic form, and direct efflux of metal out of the cell (Tripthi *et al*., 2005).

Cadmium enters the bacterial cells as a toxic alternative substrate for the cellular Mn^{2+} transport system in gram-positive bacteria or for the Zn^{2+} transport system in gram-negative bacteria. Mechanisms exist that cause changes in the membrane manganese transport systems so that Cd^{2+} is no longer taken up. This makes the cells impermeable to cadmium. Moreover, several efflux mechanisms exist that confer cadmium resistance. The *cadA* system confers resistance to Cd^{2+} and Zn^{2+}. It codes for an energy-dependent efflux mechanism. Some of the cadmium-tolerating cells have developed mechanisms for binding of cadmium by surface factors or intracellular binding factors. This resistance mechanism is important for removal of cadmium from the environment. In addition, there are several examples of Cd^{2+}-binding systems like precipitation on the cell surface, precipitation as $CdHPO_4$ and binding of Cd^{2+} by thiols. The cell surface is the most important location for precipitation of heavy metals in general (Eliora *et al.*, 1992). Cadmium has been shown to bind to capsular material in *Arthrobacter viscosus* and in *Klebsiella aerogenes* (Scott & Palmer, 1990). A *Citrobacter* mutant isolated from metal-polluted soil accumulates Cd^{2+} as insoluble cell-bound $CdHPO_4$ during growth in the presence of Cd^{2+} and glycerol. There is no conclusive evidence for microbial transformation of cadmium.

Some examples of cadmium resistant bacteria and the possible mechanisms for conferring cadmium resistance are presented in Table: 1.

Table: 1 Cadmium resistant Bacteria and the mechanisms involved in providing resistance.

Microorganisms	Location of Resistance	Suggested mechanism	Other metals
Staphylococcus aureus	R plasmid Chromosome R plasmid	Efflux (*CadA*) Efflux Binding (*CadB*)	Zn Hg
Bacillus subtilis	Chromosome	Permeability	Hg
Pseudomonas aeruginosa	R-plasmid	Efflux	NA
Pseudomonas putida	Plasmid	Binding	NA
Klebsiella pneumonia	Plasmid	Efflux	Hg
Klebsiella aerogenes	Chromosome	Binding(capsule)	NA
Alcaligenes autrophus	Plasmid	Binding	Zn, Co
Citrobactor	Chromosome	Precipitation as$CdHPO_4$	NA
Protus mirabilis	Chromosome	Binding (Envelop)	NA
Arthrobactor viseosus	Chromosome	Binding(EPS)	NA
Rhodococcus fascians	Plasmid		As

Many efforts have been committed to the isolation of heavy metal-resistant bacterial population during the past years. A population of metal resistant bacteria will be capable of growing in the metal contaminated site and thus will be more resilient to stress. Therefore the present investigation is aimed at isolation of cadmium resistant bacteria from various industrial sites that can be effectively used for the removal of heavy metals from waste water.

LITERATURE REVIEW

After mercury, cadmium (Cd) is the most toxic heavy metals and has already caused several environmental problems including the most painful affliction, *Itai-itai* (Matsuda *et al.*, 2003). It has been integrated as the majority hazardous metal in the US Environmental Protection Agency's list of priority pollutants (USEP, 1992). Its reported concentration ranges average 0.1µg $liter^{-1}$ or less in seawater, less than 0.01 to 0.06 µg $liter^{-1}$ in unpolluted fresh waters and up to 5mg $kg^{-1}$and 0.03 to 1 mg kg^{-1} in inland and marine sediments, respectively. Cadmium is

non degradable in nature and hence once released to the environment, stay in circulation. Cadmium (Cd) is highly toxic heavy metal with a widespread use in industry. All over the world many areas are cadmium polluted and present a risk to people and environment. The syndromes due to cadmium poisoning at different trophic levels are too, including the most painful *Itai-itai* disease. To eliminate environmental effects, emissions of cadmium should be reduced from smelters, incinerators, sewage sludge applied to the land, phosphate fertilizers and cadmium containing manures.

Conventional methods for the removal of heavy metals include chemical precipitation, ion-exchange method, complexation, electrochemical cells and reverse osmosis (Rao *et al.,* 2010). Chemical precipitation of metals is achieved by the addition of coagulants such as alum, lime, iron salts and other organic polymers (Ahalya *et al.,* 2003). Ion exchange and adsorption can be the result of three different interactions, which act in combination: The main contribution for free metal ions (which are highly soluble in water) is usually the attraction of the sorbats (metal ions) to the sorbent (biomass). Additionally hydrophobic expulsion may also play a role. Complexation plays an important role in both metal-ligand and sorbate-sorbent interactions. Complexes can be neutral, positively charged or negatively charged. The number of coordinating atoms in the ligands that are directly attached to the central atom is the coordination number. Electrodialysis is another membrane process for the separation of ions across charged membranes from one solution to another using an electric field as the driving force. Application of an electrical potential between the two electrodes causes a migration of cations and anions towards respective electrodes. Because of the alternating spacing of anion and cation permeable membranes, cells of diluted and concentrated salts are formed. Formation of metal hydroxides has some drawback like it clogs the membrane. Reverse osmosis is a process where heavy metals are separated by a semi-permeable membrane at a pressure greater than osmotic pressure caused by dissolved solids in wastewater (Chatterjee, 2006). It is very exclusive. On the other hand such physicochemical methods suffers from many disadvantages like they are expensive, lack the required specificity compulsory for treating target metal against a background of competing ions, unpredictable metal removal, high reagent requirement and generation of toxic sludge, which are often difficult to dewater and need extreme caution in their disposal. Moreover such approaches are not relevant to cost effective remediation of large-scale subsurface contamination in situ.

Microbial flora detoxifying cadmium is of great importance in dealing with cadmium pollution. Bioremediation encompasses technologies that accelerate natural processes for degrading heavy metals and thereby provide a good cleanup strategy. Resistance to cadmium by a variety of bacteria has been quite well understood. Such heavy metal resistant bacteria are important in removal of heavy metals from contaminated site. It is generally assumed that the exposure to metals leads to the establishment of a tolerant or resistant microbial population. Many efforts have been devoted to the isolation of heavy metal-resistant bacterial strains during the past years. Chovanova *et al.,* (2004) studied cadmium resistant bacterial community isolated from sewage sludge contaminated by cadmium ions. Among bacteria from bacterial community short cadmium resistant gram-negative rods were dominant. Biochemical tests assigned the eight isolates to six bacterial species, *Alcaligenes xylosoxidans, Comamonas testosteroni, Klebsiella planticola, Pseudomonas putida, Pseudomonas fluorescens,* and *Serratia liquefaciens*. *Pseudomonas* species resistant to cadmium (3-11mM) has been isolated by Hussein *et al.,* (2004). Both Gram positive and Gram negative bacteria has been shown to possess resistance against heavy metal (Abdelatey *et al*., 2011) and can be isolated from different sources. Cadmium resistant bacteria were isolated from water and soil sediment (Abd-Elnaby *et al*., 2011) and were found to have a minimal inhibitory concentration of 60ppm. The most effective isolate was able to accumulate Cd^{2+} concentration of 23.3mg Cd^{2+}/g and was recognized as *Vibrio harveyi*. It was noted that the occurrences of cadmium resistant bacteria in sediments samples were higher than water sample. In another study, the tolerance of *Saccharomyces cerevisiae* against cadmium was found to be upto 500ppm (Damodaran *et al*., 2011). Some of the resistant strains are able to remove the heavy metal even in the presence of the industrially used metabolic inhibitors (Luo *et al.,* 2011) or pesticides like BHC, 2,4-D, mancozeb, as well as phenolic compounds like cresol, phenols, catechol, and resorcinol (Wasi *et al*., 2010).Cadmium-resistant bacterial isolates were able to remove cadmium from solution and the efficiency of cadmium removal correlated with the amount of additionally synthesized proteins in the cell fractions (Sarin *et al.,* 2004).

Since immobilized microorganisms provide a potential system for the treatment of metal contaminated waters, many studies have been conducted using such immobilized cultures. Cells of *Alcaligenes eutrophus* were immobilized on a flat sheet reactor made of composite membranes of polysulfone with inorganic fillers, through which a nutrient solution was passed.

In 72 hours of incubation about 90% of the cadmium was removed from a solution containing 320 ppm (Diels, 2004). *Alcaligenes eutrophus* was also used for the biotreatment of soil in a slurry reactor. The soil contained about 20 ppm of cadmium 50% of which was removed in a two-step reaction. Mahvi and Diels (2004) studied the biological removal of cadmium by *Alcaligenes eutrophus* CH34 by immobilizing the cells on polysulfone membrane and observed that the efficiency for cadmium removal was over 99 percent. The immobilized bacteria induced the metal precipitation and metal crystals. Sinha *et al*., (2009) isolated a cadmium resistant *Pseudomonas aeruginosa* strain KUCd1. The isolate exhibited high cadmium accumulation under in vitro aerobic condition. The strain showed a vital ability to remove 89% of the soluble cadmium from Cd-amended industrial wastewater. Another strain of *Pseudomonas* (*Pseudomonas fluorescens G7*) was implicated for bioremediation of soil and it could remove 16.7% of cadmium after incubation of 2 weeks (Wasi *et al.,* 2010).

Heavy metal contamination of discharged wastewater is a constant area of concern. Globally, there is the challenge to remediate hazardous metal-containing water and soil. Since legislations and guidelines have created strict distribution and discharge limits, the removal of heavy metals from domestic and industrial waters has necessitated the need for economically viable and effective technologies, in order to supply safe drinking water and discharge waste water. At present, no single technology for heavy metal remediation (chemical remediation, phytoremediation or microbial remediation) is without some form of merits and demerits. Due to the enormous benefits and drawbacks of each of the existing remediation technologies, there is a need for the implementation of an integrated remediation technology which can have great potential. This can be attained through further heavy metal remediation research, which will help offer enormous environmental and cost benefits.

DESCRIPTION OF TOPIC

Heavy metals are toxic and pose a threat to human being. They persistently get accumulated in the food chain thus contaminating the environment. They may decrease metabolic activity and diversity. Not only this, they also affect the quantitative and qualitative structure of microbial communities. Bioremediation offers the possibility to destroy or render harmless various contaminants using natural biological activity. This process is less expensive than other technologies that are used for clean-up of hazardous waste. Cadmium is one of the most

dangerous metal ions characterized by its high stability and toxicity. It is not degradable in nature and will stay in circulation. Cadmium binds with the essential respiratory enzymes causing oxidative stress and cancer. Cadmium contamination has been also reported particularly in soils containing waste materials from mines and soils fertilized with cadmium rich phosphate fertilizers. The present investigation will be undertaken with the aim of identifying highly efficient metal removing bacterial systems. The ability of microbial strains to grow in the presence of heavy metals will be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter. Industrial effluents should be treated for their high BOD before they are discharged into the environment. The BOD standards laid down by the Government agencies for the discharge of wastewater into the environment are 30 mg/L for discharge on inland surface and 100 mg/L for disposal. The use of bacteria for the removal of BOD has been in practice since long, but most of the inoculated bacteria are not efficient as they cannot withstand the high concentrations of heavy metals present in the effluents. The inhibitory effect of heavy metals is a common phenomenon that occurs in the biological treatment of waste water and sewage having high BOD. In the present investigation, efforts will be diverted to isolate specific novel bacteria which can survive under such extreme environment and can effectively be used for the treatment of industrial effluents.

OBJECTIVES OF RESEARCH

Many efforts have been dedicated to the isolation of heavy metal-resistant bacterial strains during the past years. A population of metal resistant bacteria will be capable of adapting in the metal contaminated site and thus will be more resilient to stress. Therefore the present investigation is aimed at isolation of cadmium resistant bacteria from various industrial sites with the following objectives

1. Isolation and enumeration of bacterial population capable of tolerating cadmium from the soil sediment.
2. Sudying the effect of cadmium on bacterial growth and determination of minimal inhibitory concentration (MIC).
3. Bioremediation of the industrial effluent/sewage using the selected isolates.
4. Molecular characterization of the potential isolates.

METHODOLOGY TO BE ADOPTED

Collection of samples

Soil samples will be collected from the fields irrigated with waste water and from the soils of the Industrial area.

Isolation of bacteria resistant to cadmium

For the isolation of bacteria resistant to cadmium, heavy metal incorporated media will be used. Bacterial isolates will be screened on Luria Bertani (LB) media agar plates supplemented with cadmium sulphate by the standard pour plate techniques.

Determination of Minimal Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MIC) of the cadmium ion will be determined by the plate-dilution method as described by Malik and Jaishwal (2000). Stock solution of cadmium sulphate will be prepared in distilled water and sterilized by autoclaving at 121^0C for 15 min. The heavy metal solution will be added to Luria Bertani agar in various concentrations (10-1000mM) and then spot inoculated with the selected isolates .The plates will be inoculated at30^0C for 48hours.The concentration of the metal which will permit growth and beyond which there will be no growth will be considered as the MIC of the metal against the strain tested. MIC of the other heavy metals like mercury, copper and lead will also be tested by the same method using the stock solution of mercuric chloride, copper sulphate and lead sulphate respectively

Determination of optimal growth conditions

The optimal pH and temperature of the selected isolates will be determined .The isolates will be grown in LB medium with different pH values (5, 6, 7, 8, and 9) and incubation will be carried out at different temperatures 25^oC, 30^oC, 37^oC, and 40^oC.

Determination of antibiotic resistance

Heavy metal resistance has been shown to be associated with antibiotic resistance. So the selected isolates will be screened for their resistance to different antibiotics on nutrient agar by the disc diffusion method.

Screening of the selected isolates for the presence of plasmids

The selected isolate will be screened for the presence of the plasmid DNA using the standard alkaline lysis method.

Cadmium detoxification by the cadmium resistant isolates

Selected isolate will be grown in LB medium supplemented with 100ppm cadmium sulphate. The flask will be incubated on a shaker for 120 hours at 30^{o}C. Sample will be withdrawn aseptically at an interval of 24hours and the absorbance will be read at 660nm. One ml sample will be centrifuged at 13000rpm for15 minutes. Cadmium concentration will be measured from the supernatant by the atomic absorption spectrometry.

Sewage bioremediation using selected isolate

Sewage water will be collected, pH will be adjusted to 7.2, sterilized and one ml of 12 hours grown culture will be added to the experimental sets containing 50ml wastewater. The samples will be withdrawn aseptically at an interval of 24hours and will be centrifuged at13000rpm for 15minutes.Cadmium concentration will be measured from the supernatant by the atomic absorption spectrometry. Cadmium removal efficiency will be calculated by taking difference of the metal content of the culture at time zero and at the time of sampling.

Molecular characterization

Molecular characterization will be done according to 16S ribosomal DNA sequencing.

PROPOSED/EXPECTED OUTCOME OF RESEARCH

The disposal of heavy metals from various industrial operations into aquatic ecosystems and soil sediments gained the focus of ecologist from past few decades. Pollutants that pose great threat to our environment include cadmium, lead, chromium, mercury, uranium, selenium, zinc and nickel. These toxic materials may be derived from mining operations, refining ores, sludge disposal, fly ash from incinerators, the processing of radioactive materials, metal plating, or the manufacture of electrical equipment, paints, alloys, batteries, pesticides or preservatives. Physico-chemical methods of heavy metal removal such as chemical precipitation, electroflotation, ion exchange, reverse osmosis and adsorption on activated charcoal etc. are generally not cost effective and unsuitable in places where the heavy metal concentrations are low but still above the permissible limits. Methods employing a biological agent show great promise in achieving the above goal. Metal removal may be a simple adsorption of it on to the surface of the organism i.e., biosorption. Another mode of metal removal is bioprecipitation through the precipitant ligands (inorganic phosphate, sulfide) generated by the organism. The metal may be precipitated as highly insoluble metal phosphate or sulfide.

In the present investigation, the isolation of heavy metal resistant bacteria will be done from the soil samples. These isolates will be tested for the presence of plasmid using the modified alkaline lysate method. Plasmid-encoded genes embody a pool of mobile DNA that can contribute to the genetic adaptation of microbial communities. The data obtained shall give clues regarding the prevalence of plasmids in heavy metal resistant bacteria populations residing in the industrial areas. This could be used as an indicator to determine the degree of bacterial activity in eliminating toxic metals from the environment. The resistance of these cadmium resistant bacteria to several heavy metals will also be studied. This enthuses to recommend their potential to be exploited in bioremediation of mixed wastes and wastes having high BOD, since the industrial effluents contain large amounts of heavy metal contaminants which are difficult to be tolerated by these microorganism and therefore, it affects the degradation of organic compounds indirectly. Further studies related to understanding the mechanism of heavy metal tolerance will be useful in developing practical means for environmental cleanup.

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